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RNA Editing of Wheat Mitochondrial ATP Synthase Subunit 9: Direct Protein and cDNA Sequencing

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RNA editing of subunit 9 of the wheat mitochondrial ATP synthase has been studied by cDNA and protein sequence analysis. Most of the cDNA clones sequenced (95%) showed that editing by C-to-U transitions occurred at eight positions in the coding region. Consequently, 5 amino acids were changed in the protein when compared with the sequence predicted from the gene. Two edited codons gave no changes (silent editing). One of the C-to-U transitions generated a stop codon by modifying the arginine codon CGA to UGA. Thus, the protein produced is 6 amino acids shorter than that deduced from the genomic sequence. Minor forms of cDNA with partial or overedited sequences were also found. Protein sequence and amino acid composition analyses confirmed the results obtained by cDNA sequencing and showed that the major form of edited *atp*9 mRNA is translated.

INTRODUCTION

RNA editing is a biological phenomenon consisting of the modification of the genetic message leading to the creation of new codons or to the alteration of reading frames. Several mechanisms have been described such as the addition/deletion of uridine residues in trypanosome mitochondria (Simpson and Shaw, 1989; Benne, 1990) and the addition of guanosine residues on paramyxovirus RNA (Cattaneo et al., 1989). In the case of higher plant mitochondria, RNA editing is found in several species and has been described for several protein coding genes (Covello and Gray, 1989; Gualberto et al., 1989; Hiesel et al., 1989; Lamattina et al., 1989). The post-transcriptional modifications, observed by RNA or cDNA sequence analysis, occur mainly by C-to-U transitions, similar to the situation described for apolipoprotein B in mammalians (Higuchi et al., 1988). The mechanism by which the C-to-U changes occurs is unknown. In a recent paper (Graves et al., 1990), we showed that when performing the partial protein sequencing of the mitochondrial subunit 9 of ATP synthase (ATP 9), we found that some residues differ from those encoded for by the mitochondrial *atp*9 gene. The differences are explained by assuming C-to-U transitions at the mRNA level. Thus, we showed that mRNA modifications by RNA editing are reflected at the translational level.

In this paper, we extend these observations to the cDNA sequence of *atp*9, and we demonstrate the presence of partially modified mRNA molecules. One C-to-U conversion transforms an arginine codon into a stop codon, shortening the protein to the "standard" size when compared with other mitochondrial ATP 9. The analysis of subunit 9 by peptide sequencing and amino acid composition confirms these results.

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RESULTS

cDNA Sequence Analysis

We have previously shown, by partial peptide sequencing of ATP 9, that some amino acid residues are not encoded by the mitochondrial gene (Graves et al., 1990). The differences observed can be explained by RNA editing concerning C-to-U changes as described for higher plant mitochondria (Covello and Gray, 1989; Gualberto et al., 1989; Hiesel et al., 1989). In this paper, we extend our first results to the analysis of cDNA sequences and we verify the predicted primary structure of the protein by peptide sequencing.

Two primers flanking the coding region of *atp*9 were used to amplify the cDNA sequences by polymerase chain reaction (PCR). The cDNA thus obtained was cloned and the recombinants were isolated for double-stranded DNA sequencing experiments with T7 DNA polymerase. More than 50 clones were analyzed. Most reflected completely edited sequences involving eight C-to-U transitions scattered all over the coding region. cDNA gel sequences

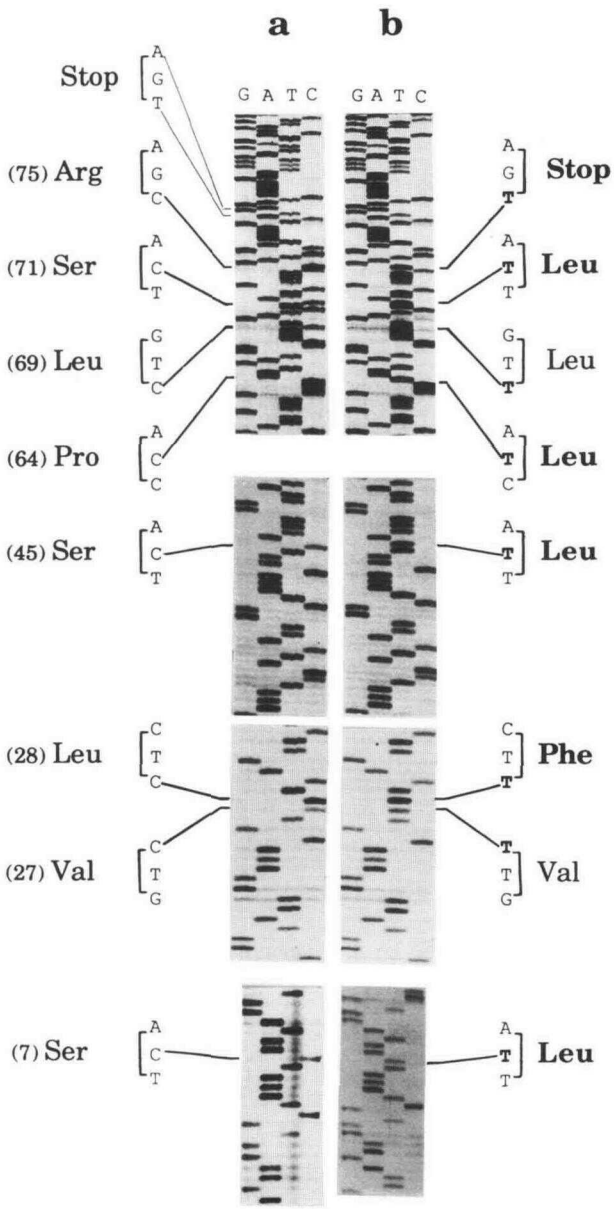


Figure 1. Sequencing Gel Autoradiography of Four Regions of the Genomic DNA and the Major cDNA *atp*9 Clones.

Only the codons involved in editing are shown and triplets are designated by square brackets. The respective amino acids are shown and the residue number in the protein is in parentheses. The T residues obtained by editing are shown in bold type. a, genomic DNA; b, cDNA.

showing the mRNA edited sites were compared with the sequence obtained from genomic clones, as shown in Figure 1. Three editing events corresponded to a UCA-to-UUA transition leading to a serine to leucine change at positions 7, 45, and 71 (Figure 1). At position 28, a CUC (Leu) codon was converted to a UUC (Phe) codon, and at position 64 a CCA (Pro) codon was changed to CUA (Leu). Two C-to-U transitions gave no change in the predicted protein sequence (silent editing). In fact, at positions 27 and 69 the codons GUC and CUG were changed to GUU and UUG, which also code for Val and Leu, respectively. A striking observation is the editing occurring at position 75 where the arginine codon CGA was changed to the stop codon UGA. This modification led to a predicted protein 6 residues shorter than the one encoded for by the gene. These results are summarized in Figure 2.

We observed partial edited sequences and some editing points outside of the regions described above. They represented nearly 10% of the sequenced clones. One C-to-U editing event occurred at position 21, where a GTT (Val) triplet was observed instead of a GCT (Ala) triplet as shown in Figure 3A. cDNA molecules edited only at position 7 were also found. Interestingly, all of the cDNA clones analyzed contained the codon 7 in the edited form. A partial edited form is illustrated in Figure 3B in a position where two consecutive editing events occur. Codon 27 is not edited and codon 28 is present in the edited form. Because the editing at codon 27 is silent, this kind of situation has no consequence at the protein sequence level. An unexpected U-to-C transition at position 68 was deduced from a completely edited cDNA clone (Figure 3C). This is a silent change in which a Phe residue is conserved.

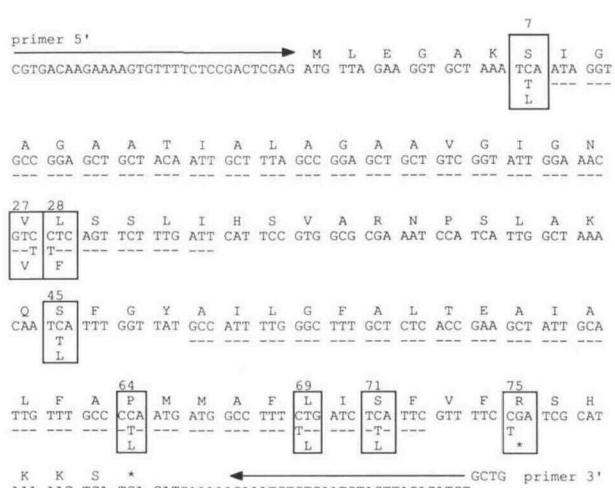


Figure 2. Nucleotide and Amino Acid-Deduced Sequences from the *atp*9 Gene and cDNA Clones.

The complete genomic sequence and only the major edited bases from cDNA are shown. Boxes signal the changes produced after editing. Asterisks mark the genomic and the editing-created stop codon. Arrows show the position of primers used in PCR amplification experiments. Dashed underlines indicate membrane-spanning regions according to Ooi et al. (1985).

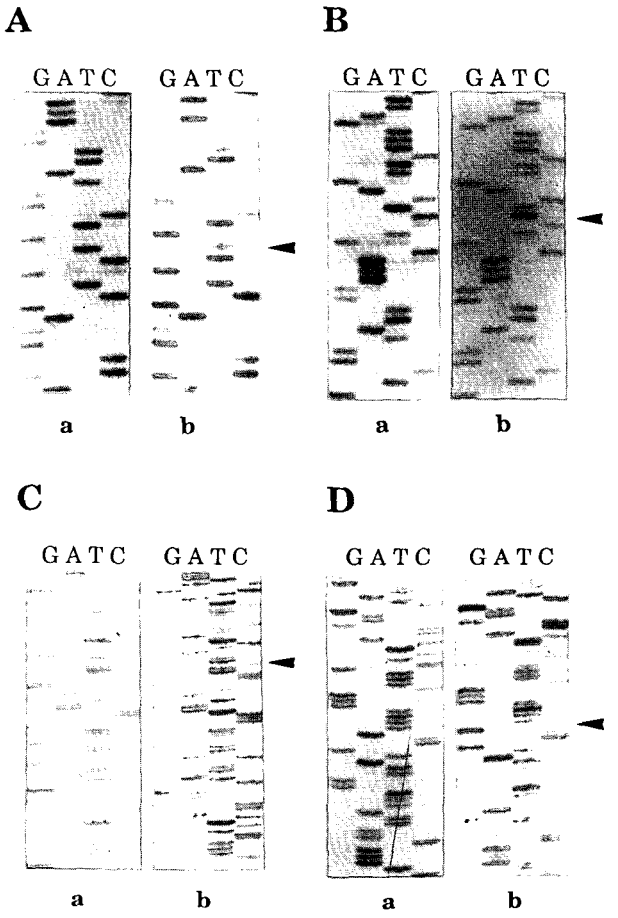


Figure 3. Sequence Gel Autoradiography of Different Editing Events Found in *atp9* cDNA Clones.

(A) Editing at codon 21.
(B) Partial editing at codons 27, 28.
(C) Reverse editing at codon 68.
(D) A-to-G transition at codon 50.
These clones represent 5% of recombinants sequenced. a, genomic sequences; b, cDNA sequences. The edited nucleotides are indicated by arrows.

Whether this "reverse transition" is a consequence of a highly active editing region (four events on 36 nucleotides) remains to be established. Recently, Schuster et al. (1990a) found a U-to-C transition on apocytochrome *b* cDNA from *Oenothera berteriana*. One clone shows an additional A-to-G transition at position 50 in which the triplet ATT(Ile) is replaced by GTT(Val) (Figure 3D).

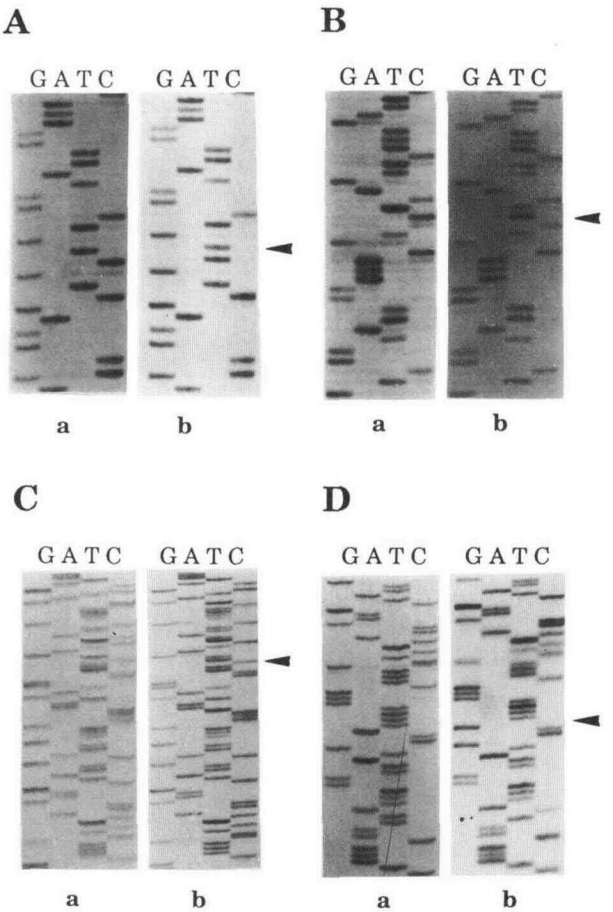
Amino Acid Analysis

To determine whether the edited genetic message of *atp9* was reflected in the translation product, we purified ATP 9 (Graves et al., 1990), and the amino acid composition was done after total hydrolysis in 6 M HCl (Velours et al., 1987). The results are shown in Table 1 (column b) and are compared with the theoretical composition of the deduced sequences from cDNA and genomic *atp9* (Table 1, columns a and c, respectively). The genomic sequence predicts a protein of 80 residues, whereas the cDNA sequence gives a shortened protein of 74 residues. It is clear from nucleotide sequence analysis that both proteins differ in amino acid composition, especially for residues Ser and Leu. In the case of serine, the genomic sequence predicts 9 residues and the cDNA edited sequence only 4. Amino acid analysis gives an average of 4.8 residues in accordance with the cDNA codon usage. A clear-cut result was obtained for leucine where 12 residues were obtained as predicted for edited RNA instead of 9 residues when genomic (nonedited) codon usage was considered. Seven phenylalanine residues were obtained instead of 6; 1 Phe residue arose from a CUC-to-UUC transition on codon 28, which is in agreement with the results of amino-terminal sequences previously published (Graves et al., 1990). The figures corresponding to histidine, lysine, and arginine (1.0, 1.9, and 1.3 residues per molecule, respectively) are in accordance with the prediction of a protein shortened by creation of a stop codon at position 75 (Figure 2). In fact, the change of the Arg CGA codon into a UGA stop codon gives a protein lacking 2 Lys, 1 His, and 1 Arg residue.

Table 1. Amino Acid Composition of Wheat Mitochondrial ATP 9

	No. of Residues		
Amino acid	a	b	c
Asp + Asn	2	1.8	2
Glu + Gln	3	2.9	3
Ser	4	4.8	9
Gly	8	8.6	8
His	1	1.0	2
Thr	2	2.3	2
Ala	16	16.2	16
Arg	1	1.3	2
Pro	1	1.5	2
Tyr	1	1.0	1
Val	4	4.1	4
Met	3	2.0	3
Ile	7	7.0	7
Leu	12	12.0	9
Phe	7	7.0	6
Lys	2	1.9	4
Total	74		80

Columns a and c represent the number of residues of the deduced sequences obtained from cDNA and genomic coding regions, respectively. Column b shows the results obtained from three independent experiments of the amino acid analysis composition of the purified ATP 9 subunit (for details see Methods).



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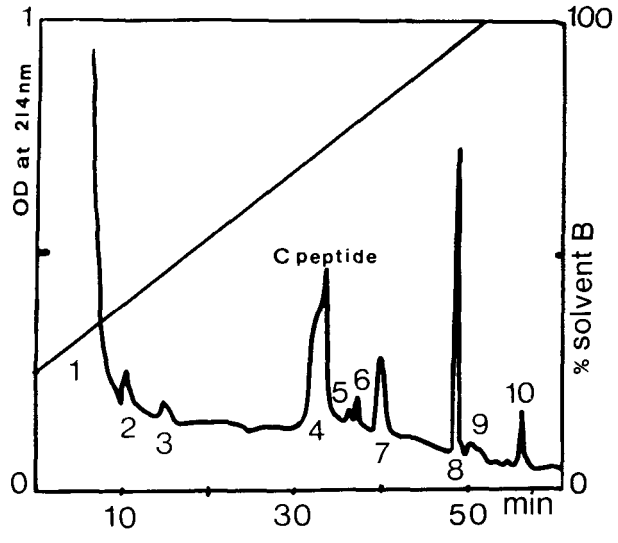
A



B

A F L I L F V (F) *

Figure 4. Isolation and Sequence of the ATP 9 Carboxy-Terminal Peptide.

(A) *HPLC chromatography of cyanogen bromide-cleaved ATP 9.*
(B) Carboxy-terminal sequence of ATP 9. Parentheses indicate a Phe residue not detected directly by sequencing. For details see Methods.

Carboxy-Terminal Sequence of ATP 9

To confirm the creation of a stop codon at position 75, we cleaved the protein at the methionine residues with cyanogen bromide. According to the predicted sequence of ATP 9, only two peptides were expected: the first is the N-terminal peptide from Leu-2 to Met-65 and the second is the C-terminal peptide from Ala-67 to Ser-80 (long) or to Phe-74 (short) if a stop codon is created by editing. The short C-terminal peptide should contain 8 nonpolar residues and the long one should have 6 additional polar and positively charged residues. We took advantage of these potential differences to isolate them. After cleavage, the mixture components were separated on an HPLC column with an acetonitrile gradient in TFA, as shown in Figure 4A. An aliquot of each peak was analyzed by thin layer chromatography on silica plates with *n*-propyl alcohol/25% NH_4OH (70/30, v/v). Only peak 1 eluting at 5 min and peak 4 eluting at 32 min were developed with fluorescamine (not shown). Peak 4, containing a pure highly hydrophobic peptide, was loaded on an automated sequenator. The amino acid sequence of peak 4 is shown in Figure 4B. It corresponds to a small peptide of 8 residues whose sequence perfectly matches the predicted sequence from edited RNA. A Leu residue instead of Ser is confirmed at position 71. A drop in signal after Val-73 in the sequenator detector clearly indicates that the peptide ends after residue 74, as predicted from the major edited message.

DISCUSSION

In this paper, we report the results of sequence analysis of cDNAs derived from the coding region of *atp*9 mRNA and the amino acid analysis and the carboxy-terminal sequence of the protein. Our results clearly showed that the *atp*9 transcripts are modified by C-to-U changes in a process called RNA editing. Eight codons are involved in RNA editing: five lead to an amino acid change, two give no modification, and one transforms an Arg codon into a

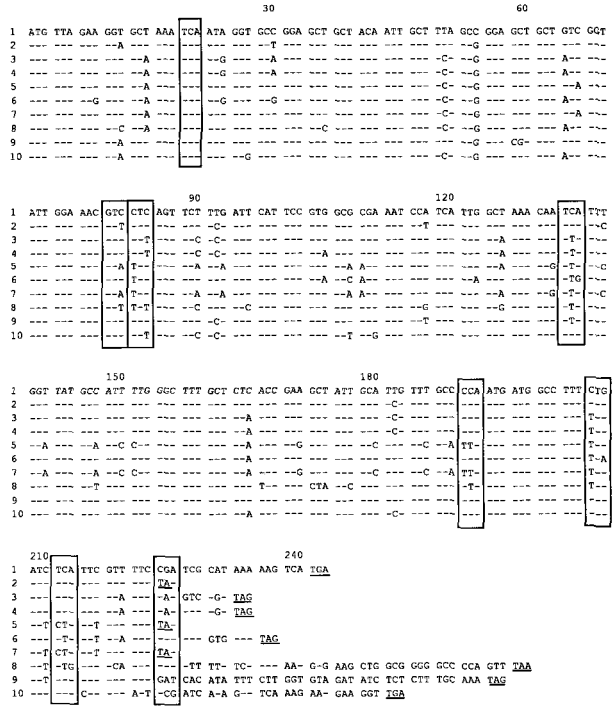


Figure 5. Sequence Comparison between *atp*9 Genes from Higher Plant Mitochondria.

1, Wheat (Bégu et al., 1989); 2, maize (Dewey et al., 1985); 3, tobacco (Bland et al., 1986); 4, petunia (Young et al., 1986); 5, pea (Morikami and Nakamura, 1987); 6, *Oenothera* (Schuster and Brennicke, 1990); 7, broad bean (Wahleithner and Wolstenholme, 1988); 8, sugar beet (Xue et al., 1989); 9, rice (Kaleikau et al., 1990); 10, sunflower (Recipon, 1990).

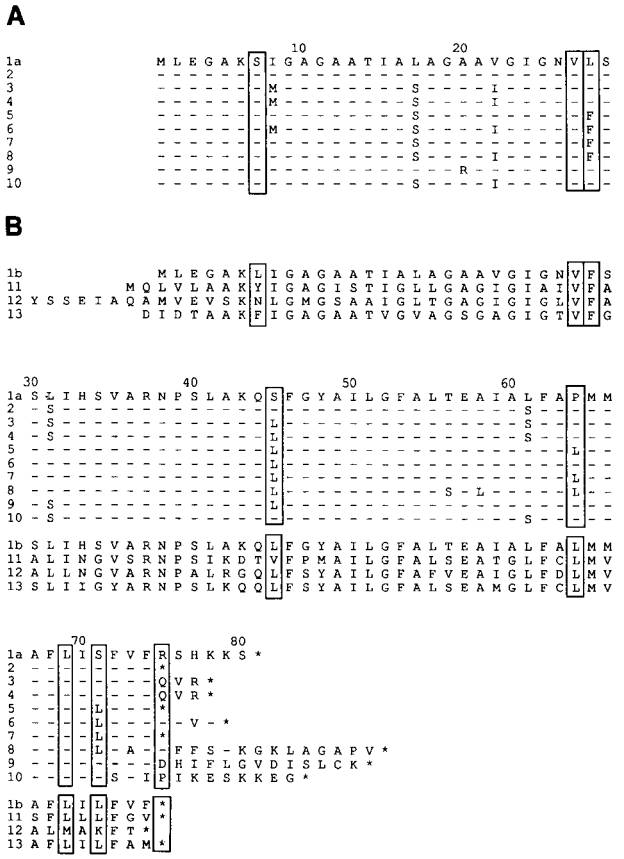


Figure 6. Comparison between ATP 9 Protein Sequences from Plants and Nonplant Organisms.

(A) Comparison of protein sequences of ATP 9 from plant mitochondria deduced from gene sequence data. 1a, wheat ATP 9 sequence deduced from gene.
(B) Comparison of direct protein sequences from: 1b, wheat; 11, yeast; 12, *Neurospora crassa*; 13, beef liver (Sebald et al., 1979). Boxes indicate the positions involved in RNA editing.

stop codon. The editing process for wheat ATP 9 represents an important modification in genetic information, considering that the gene is only 243 nucleotides long.

The mRNA modifications observed at positions 7, 28, 45, 64, 71, and 75 fit well with the hypothesis that RNA editing may be a mechanism in maintaining a high degree of similarity at the protein level, despite sequence divergence at the genomic level, as shown in Figure 5. This analysis is reinforced by comparing the wheat ATP 9 with nonplant subunit 9, as shown in Figure 6B, with the exception of position 7. By contrast, at positions 27 and 69, where codons GUC and CUG are changed to GUU and UUG, respectively, no modification of the amino acid residues is involved (silent editing). This situation raises the question of why plant mitochondria conserve editing events not leading to changes at the translational level. A possible interpretation is that RNA editing is more than a "repair" mechanism for protein sequence conservation and could be involved in some step of the control of genetic expression. When comparing the nucleotide sequence of different plant *atp*9 genes (Figure 5), it is clear that some have "corrected" the gene to the edited form, as is the case at nucleotides 134 and 205 and to a lesser extent at nucleotides 81, 82, 191, 212, and 223. Conversely, by comparing genomic sequences, it should be possible to predict the positions where editing of *atp*9 transcripts should occur in some plant mitochondria. At position 7, all of the *atp*9 sequences obtained so far have the same codon, suggesting that the C-to-U conversion at this position has been conserved in plant mitochondria. Interestingly, all sequenced clones were edited at residue 7, suggesting that this is a particularly active RNA editing environment. The molecules edited at this particular position could be a precursor form of the edited RNA.

At position 75, the codon CGA encoding for arginine is changed to a UGA stop codon. This is an important observation because this situation "corrects" the message to avoid the sequence heterogeneity observed at the carboxy termini of plant ATP 9 by deducing the protein sequence from the genomic data (Figures 5 and 6A). In fact, several proteins have a genomic stop codon or a glutamine codon able to give UAA or UAG stop codons by RNA editing at this position. We postulate that codons CAA, CAG (Gln), and CGA (Arg) are, by C-to-U editing, potential stop signals in higher plant mitochondria.

Some RNA editing besides that described above was also observed, such as at position 21, where an alanine (GCU) is changed to a valine (GUU) codon, suggesting that the C-to-U change may not be an accurate process. At this position, protein sequence data do not show a valine residue. It is also possible that the level of the altered protein is not high enough to be detected by the sequencing procedure. More intriguing is the observation of an A-to-G change at position 50, where a codon AUU (Ile) becomes GUU (Val). It is difficult to conclude by comparisons with other *atp*9 sequences whether it is a physiological process because all genes contain an ATT or ATC triplet. However, when comparing position 22, where the wheat gene has a GTC (Val) triplet, five out of 10 mitochondrial plant genes have the ATC (Ile) sequence, suggesting a potential A-to-G transition. This event does not seem to be artifactual because we also observed A-to-G changes with different frequencies related to apparent editing deficiencies when we analyzed the same sequences in an alloplasmic line of wheat (D. Bégu, S. Litvak, and A. Araya, manuscript in preparation). Further work is necessary to know whether this particular editing is functional in plant mitochondria.

Three residue changes out of six occur in the *trans*-membrane portion of the protein (Figure 2). Particularly

interesting is the modification Pro to Leu. In fact, the presence of a proline residue at position 64 should induce a kink in the α-helix (Barlow and Thornton, 1988; Dempsey, 1990), and the editing could be considered as a correction process. It is not clear whether the presence of a proline residue may affect the activity of ATP synthase. The extra-membrane region is edited on codon 45 (Ser to Leu), which does not seem important for activity, whereas the highly conserved sequence Arg-Asn-Pro-Ser-Leu, essential for ATP synthase function (Willson and Nagley, 1987), is not affected.

Despite the specificity of the editing mechanism, no consensus sequence can be clearly derived in the vicinity of each edited site. Nevertheless, the closest 5′ neighbor nucleotides at the eight modified positions are pyrimidines; five are T and three are C. We have analyzed 63 editing sites occurring in coding regions and seven sites located at 5′-noncoding and 3′-noncoding regions from the plant mitochondrial genes described so far (Covello and Gray, 1989; Gualberto et al., 1989; Hiesel et al., 1989; Schuster et al., 1990a, 1990b, 1990c). Out of 70 editing positions examined for the nearest 5′ residues, we found that 47 residues are T, 17 are C, and 6 are A. No G residues have been reported at the 5′ vicinity of the editing position. These results clearly indicate a bias toward a pyrimidine as a putative requirement for C-to-U RNA editing in plant mitochondria. This is different from the results published for mammalian apolipoprotein B, where no clear 5′ nucleotide requirement seems to be necessary (Chen et al., 1990).

Genes encoding for subunits 9 and α of ATP-synthase are cotranscribed. Two molecules of 2.5 kb and 1.5 kb have been observed on RNA gel blot analysis (D. Bégu, manuscript in preparation). Sequence data obtained by cloning cDNA before PCR amplification make it possible to know whether editing events occur differentially on both transcripts. Our preliminary results indicate that both transcripts are identically edited (data not shown).

A note of caution is necessary in interpreting the results obtained by cDNA sequence analysis. In fact, three steps of DNA polymerization are involved in this technique: cDNA synthesis from mRNA by reverse transcriptase, second-strand DNA synthesis and PCR amplification by Taq DNA polymerase, and sequencing by T7 DNA polymerase. At each step, incorrect incorporation of precursors may lead to misinterpretation of results. We have overcome this eventuality by correlating our cDNA sequencing results with amino acid analysis and protein sequencing. In our hands, PCR amplification does not seem to affect the fidelity of the products. The sequence of the 32 amino-terminal residues of ATP 9 (Graves et al., 1990) and the sequence of the carboxy-terminal peptide clearly confirm that the editing of *atp*9 transcripts is a physiological process in plant mitochondria. The editing of codons 7, 28, 45, 64, 71, and 75 is, thus, directly demonstrated and the overall process confirmed by amino acid composition analysis.

METHODS

Isolation of Mitochondrial RNA

Wheat embryo mitochondria and mitochondrial DNA were prepared as previously described (Ricard et al., 1986). Mitochondrial RNA was prepared from purified *Triticum aestivum* embryo mitochondria by phenol extraction and LiCl precipitation (Maniatis et al., 1982). Seventy micrograms of mitochondrial RNA were resuspended in water, treated with 42 units of DNase I in the presence of 18 units of RNasin, phenol extracted, and ethanol precipitated.

cDNA Synthesis and PCR Amplification

*T. aestivum atp*9 cDNA was obtained by using a cDNA synthesis kit (Amersham Corp.) following the protocol provided by the manufacturer. First-strand synthesis was performed on 1 μg of mitochondrial RNA with avian myeloblastosis virus reverse transcriptase and primed with the 3′ primer GTCGACATCTGTAAGTACATTCACACATTTC. This primer carries a SalI recognition site at the 5′ end and is located 10 nucleotides downstream of the genomic TGA stop codon (Bégu et al., 1989). The second-strand synthesis was primed with a 31-mer CGTGACAAGAAAAGTGTTTTCTCGACTCGAG located upstream of the ATG initiation codon. This primer has an XhoI restriction site. Elongation was carried out with *Escherichia coli* DNA polymerase I and RNase H.

One-tenth of the cDNA thus obtained was amplified by PCR in a Genofit apparatus with the primers used in cDNA synthesis. The 50-μL PCR mixture contained 10 mM Tris-HCl, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM each dATP, dTTP, dGTP, and dCTP, 1 μM each primers, and 1.2 units of Taq DNA polymerase (Promega Biotec). The first cycle included incubation at 95°C for 5 min before the addition of the enzyme, then 2 min at 55°C and 2 min at 70°C. This step was followed by 30 cycles of 1 min at 95°C, 2 min at 55°C, and 2 min at 70°C. PCR products were analyzed on agarose gel. Amplified products were phenol extracted and precipitated three times with ethanol after adjusting to 2.5 M ammonium acetate.

cDNA Cloning and Sequencing

The amplified cDNA was digested with SalI and XhoI endonucleases and ligated to a SalI linearized Bluescribe vector. Transformation of *E. coli* DH5α cells was carried out as described (Hanahan, 1983). Positive clones were screened by the blue/white color selection on ampicillin plates. Recombinant plasmids were prepared by the boiling method (Maniatis et al., 1982). cDNA sequencing reactions were carried out on recombinant plasmids with T7 or T3 primers using a T7 DNA polymerase sequencing kit (Pharmacia LKB Biotechnology Inc.) and α-thio-^{35}S-dATP (600 Ci/mmol) (Amersham Corp.). Reaction products were run on 6% acrylamide gels, followed by autoradiography.

Protein Purification and Amino Acid Analysis

ATP 9 was purified from *T. aestivum* mitochondria as described (Graves et al., 1990). Amino acid composition of ATP 9 was determined according to Heinrikson and Meredith (1984). The protein was hydrolyzed in 6 M HCl for 24 hr, 48 hr, and 72 hr at 110°C. The phenylthiocarbamoyl derivatives were analyzed by HPLC chromatography on an Ultrasphere ODS (Altex) column (4.6 × 250 mm) at 52°C (flow rate 1.2 mL/min). Separation was achieved with 50 mM sodium acetate, pH 6.5, as solvent A and 100 mM sodium acetate/acetonitrile/methanol (50/40/10, v/v/v) as solvent B. A multilinear gradient was developed as described by Velours et al. (1987).

Peptide Isolation and Sequencing

Cyanogen bromide treatment of purified ATP 9 was carried out according to Gross (1967). The peptides obtained were fractionated by HPLC chromatography on an ODS column (Scientific Glass Engineering PTY. LTD., Australia) (4 × 100 mm, 3 μm) with a linear 33-min gradient from 20% to 100% in solvent B. The primary solvent (A) was aqueous 0.1% TFA and the secondary solvent (B) was 0.1% TFA in acetonitrile/H_2O (90/10, v/v). Detection was monitored at 214 nm. Automated sequence analysis of the peptides was performed in an Applied Biosystems gas phase sequenator.

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